

# Autophagy-enhancing drug carbamazepine diminishes hepatocellular death in fibrinogen storage disease

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## Abstract

Fibrinogen storage disease (FSD) is a rare autosomal-dominant hereditary disorder characterized by hypofibrinogenemia and accumulation of fibrinogen aggregates within the hepatocellular endoplasmatic reticulum (ER). Some FSD patients present with elevated amino-transferases and fibrosis/cirrhosis similar to alpha-1-antitrypsin deficiency (ATD), also an ER storage disease. Pharmacological stimulation of autophagy has been shown to mediate clearance of protein aggregates and halt progression of liver fibrosis in *in vivo* models of ATD. Our aim was to evaluate the presence of autophagy and a possible response to autophagy-enhancing therapy in patients with FSD.

Hepatic fibrosis was assessed by transient elastography in 2 newly identified FSD families with fibrinogen Aguadilla and Brescia mutations, encompassing 8 affected members. Available liver biopsies were assessed for autophagy. Two patients, who had had elevated alanine amino-transferase levels (2–5 above upper limit of normal), were treated with the autophagy enhancer carbamazepine (CBZ).

Transient elastography did not show evidence of significant fibrosis in any affected family members. Quantitative electron microscopy of one patient showed a 5.15-fold increase of late stage autophagocytic vacuoles compared to control livers. CBZ at low anticonvulsive treatment levels led to rapid normalization

of alanine-aminotransferase and decrease of caspase-cleaved and uncleaved cytokeratin-18 fragments (M30 and M65). These effects reversed after discontinuation of treatment.

Response to CBZ may be mediated by pharmacologically enhanced autophagy resulting in reduction of aggregate-related toxicity in FSD. These results suggest clinical applicability of pharmacological stimulation of autophagy in FSD, but potentially also in other related disorders.

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## Introduction

Fibrinogen storage disease (FSD) is a rare autosomal-dominant hereditary disorder characterized by hypofibrinogenemia and accumulation of fibrinogen precursor proteins within the hepatocellular endoplasmatic reticulum (ER). Four mutations within the fibrinogen gamma chain (FGC) gene that cause protein aggregation, ER retention, and consequent hypofibrinogenemia in heterozygous individuals have been identified to date: fibrinogen Brescia ( $\gamma 284 \text{ Gly} \rightarrow \text{Arg}$ ), fibrinogen Aguadilla ( $\gamma 375 \text{ Arg} \rightarrow \text{Trp}$ ), fibrinogen Al DuPont ( $\gamma 314 \text{ Thr} \rightarrow \text{Pro}$ ) and fibrinogen Angers ( $\gamma \text{del}346\text{--}350$ ) [1,2]. Aggregated fibrinogen is visible as small faintly eosinophilic coarse globules on hematoxylin/eosin (H&E) stained sections. Using transmission electron microscopy (TEM), a diagnostic “fingerprint-like” pattern can be seen within the ER. To date 26 individuals have been described (Supplementary data). Patients show variable severity in liver disease ranging from an inert carrier state to cirrhosis (Supplementary data).

Macroautophagy (referred to hereafter as autophagy) is a highly conserved and regulated cellular clearance mechanism [3]. Non-selective autophagy mediates metabolic adaption to different nutritional conditions and maintains cellular homeostasis. Selective autophagy clears aggregated and misfolded proteins as well as damaged organelles via cytosolic sequestration and subsequent lysosomal degradation.

**Keywords:** Fibrinogen storage disease; Fibrinogen; Cirrhosis; Elasticity imaging techniques; Autophagy.

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**Abbreviations:** FSD, fibrinogen storage disease; ER, endoplasmatic reticulum; FGC, fibrinogen gamma chain; H&E, hematoxylin/eosin; TEM, transmission electron microscopy; ATD, alpha-1-antitrypsin deficiency; CBZ, carbamazepine; IQR, interquartile range; ALT, serum alanine aminotransferase; AST, aspartate aminotransferase; INR, international normalized ratio; OM, original magnification; EVG, Elastic Van Gieson; n.t., not tested.



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In murine models of hepatic alpha-1-antitrypsin deficiency (ATD), autophagy-enhancing drugs carbamazepine (CBZ) and rapamycin reduced the hepatocellular burden of aggregated protein as well as liver fibrosis [4,5].

FSD and ATD share a similar pathogenesis: the accumulation of aggregated misfolded proteins within the endoplasmic reticulum and development of progressive fibrotic liver disease [6]. In a yeast model of FSD, autophagy contributes to endoplasmic reticulum clearance of fibrinogen Agudilla gamma chain [7]. Here we characterize two newly identified FSD families and evaluate the effects of pharmacological stimulation of autophagy by CBZ in affected members with elevated serum amino-transferases.

## Patients and methods

### Patients

Probands of both families, a 6-year old girl and a 5-year old boy, were referred to a tertiary institution for investigation of persistently elevated serum aminotransferases. Diagnostic liver biopsies were performed, which led to diagnoses of FSD. Tissue for paraffin sections was formalin-fixed, paraffin-processed and stained using routine procedures and stains. For quantification of autophagy, control liver tissue was obtained from 3 children with normal/nearly normal diagnostic liver biopsies and an 8-month old infant, who underwent liver transplantation for severe ATD (Supplementary data). Tissue for TEM was fixed in glutaraldehyde (patient F2 III-1, control patients 1-3, control ATD patient) or deparaffinized (patient F1 III-4) and processed using standard osmium tetroxide treatment, epon<sup>®</sup> resin embedding and uranyl acetate/lead citrate staining.

### Genetic testing

Genetic testing was performed for subtyping of FSD in the probands and their families. Informed consent according to the German Genetic Diagnosis Act was obtained on all members tested. Genomic DNA was isolated from EDTA blood samples. Exons 8 and 9 of the fibrinogen gamma gene were amplified and both strands were sequenced by conventional dideoxy Sanger sequencing using a GenomLab GeXP capillary sequencer (Beckman Coulter, Krefeld, Germany). Oligonucleotide primers used for amplification and sequencing were: exon 8 forward: 5'-AGGGTCAGCATGTGATGGTT-3'; and reverse: 5'-TCCACTTCCAGTTTCAAAGAA-3', and exon 9 forward: 5'-ACTGGCAATGCACCTTCGTAA-3'; and reverse: 5'-AAAAAGGAAGAACTTTTCAGAGAA-3'.

### Transient elastography

Liver stiffness was measured by transient elastography (FibroScan<sup>®</sup>, Echosens, France) using the XL probe for F1 I-2, the M probe for all other adults, and the S probe (S2 setting) for children. Ten consecutive measurements were performed on each patient and the median and interquartile ranges (IQR) were calculated. Liver stiffness measurements of <6 kPa (≤18 years) and <7 kPa (>18 years) were regarded as normal (Supplementary data).

### Carbamazepine treatment

Initiation of CBZ treatment was approved by the institutional ethics committee of Hannover Medical School and informed consent was obtained from parents of treated patients. Daily doses of CBZ were increased incrementally from 2 × 20 mg to maintenance doses of 2 × 150 mg (14.2 mg kg<sup>-1</sup> d<sup>-1</sup>, F2 II-1) and 2 × 200 mg (15.8 mg kg<sup>-1</sup> d<sup>-1</sup>, F1 III-4). Duration of CBZ treatment was 219 days for F1 III-4 and 208 days for F2 II-1.

### Cytokeratin-18 detection

For quantification of uncleaved and caspase-cleaved cytokeratin-18 within patients' sera, the M30 Aptosense and M65 ELISAs (Peviva, Bromma, Sweden) were used as described [8]. Sera from 9 healthy children (5.7–8.8 years, mean age 6.4 years) were used as control group.

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### Clinical history of probands

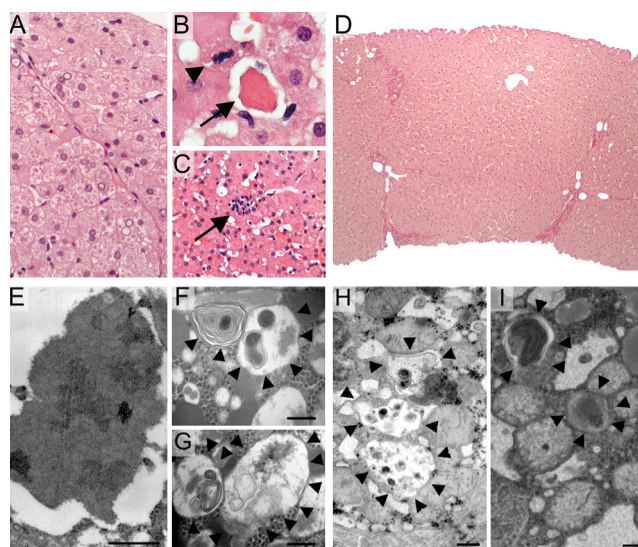
The proband of family 1 (F1 III-4), a 6-year old Caucasian girl, was found to have persistently elevated serum alanine aminotransferase (ALT) levels as well as prolonged prothrombin time on routine blood tests. The medical history was unremarkable. The proband of family 2 (F2 III-1), a 5-year old Caucasian boy, presented with repeated and prolonged episodes of epistaxis. Investigations for exclusion of a coagulopathy revealed hypofibrinogenemia and elevated ALT levels. Serologies for autoimmune hepatitis or hepatotropic viruses were negative in both patients and none of them received any medication.

### Diagnostic liver biopsies

The liver biopsies of both probands showed hepatocytes with granular cytoplasm and focal coarse irregular globules (Fig. 1A). F1 III-4's biopsy showed scattered apoptotic hepatocytes, mitoses, and necroinflammatory foci in low density (Fig. 1B and C). There was mild nodular regenerative hyperplasia and minimal collagenous expansion of portal tracts (Fig. 1D).

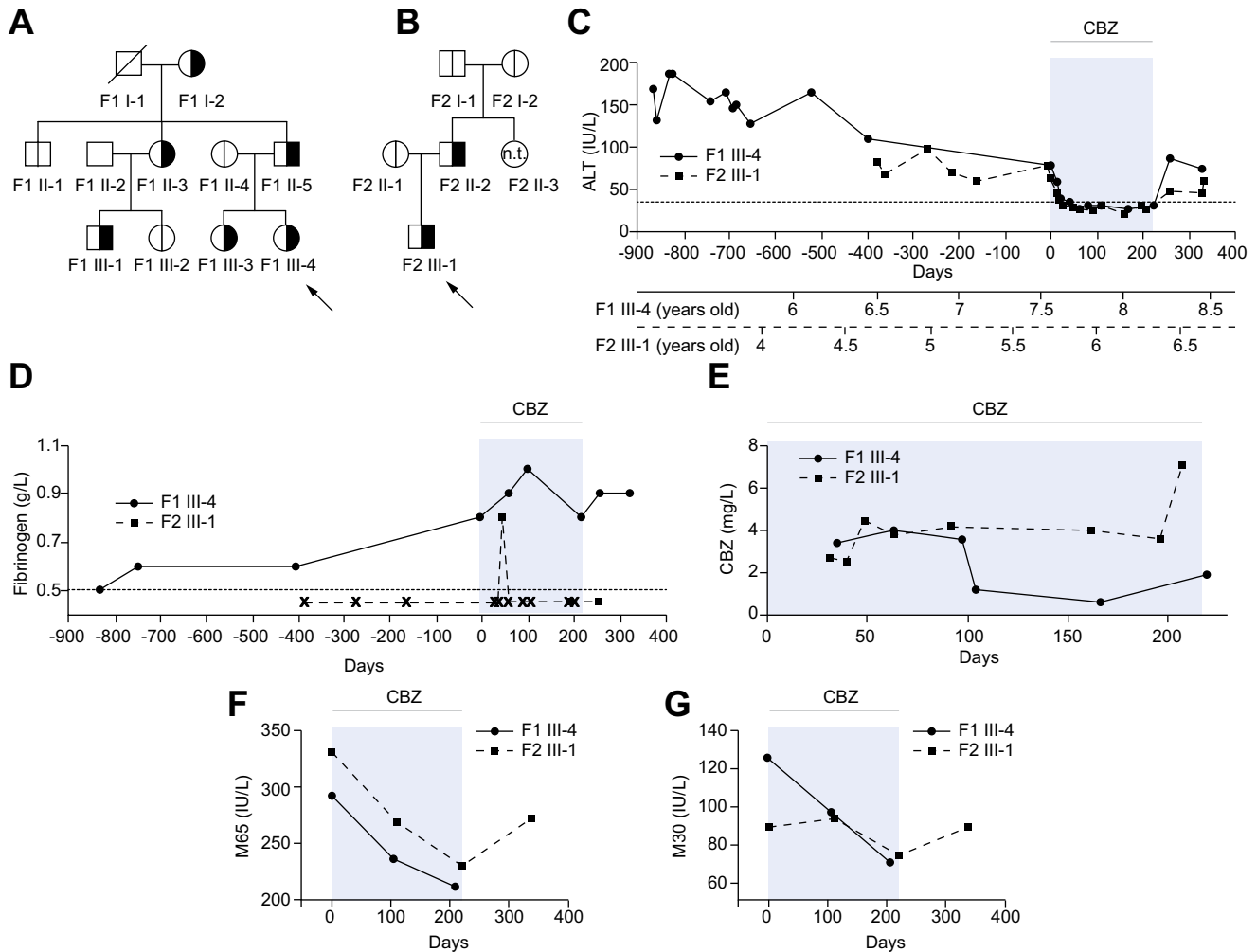
### Transmission electron microscopy

In both patients, material examined by TEM showed aggregates of curved tubular "fingerprint-like" structures within the distended ER (Fig. 1E). Poor preservation of membranous structures in the biopsy of F1 III-4 due to re-embedding of paraffin-processed tissue rendered this sample unsuitable for detection of



**Fig. 1. Histology and TEM of liver biopsies.** (A) Liver biopsy of F2 III-1 showing coarse inclusions, H&E original magnification (OM) 200x; (B) apoptosis (arrow), mitosis (arrowhead), F1 III-4, H&E OM 400x; (C) necroinflammatory activity (arrow), F1 III-4, H&E OM 100x; (D) focal periportal fibrosis, F1 III-4, Elastic Van Gieson (EVG) OM 100x; (E) aggregates show densely packed curved tubules diagnostic of FSD (F1 III-4); (F, G) late stage autophagocytic vacuoles in FSD containing aggregates as cargo (F2 III-1); (H) rare autophagocytic vacuole in a control liver (control patient 1); (I) autophagocytic vacuoles in an explanted liver of a 8-month old infant with severe neonatal ATD. Bars = 500 nm.

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**Fig. 2. Family trees of both FSD families and parameters during CBZ treatment.** (A) Family 1, fibrinogen Aguadilla, affected members in 3 generations; (B) Family 2, *de novo* fibrinogen Brescia mutation in 2 generations, (affected heterozygous individuals are highlighted by half-filled symbols, tested individuals with wild type sequences are highlighted by non-filled divided symbols, probands are indicated by arrows, n.t., not tested); (C) CBZ led to rapid normalization of ALT, which relapsed to pre-treatment levels after cessation of CBZ (dotted line = upper limit of normal); (D) hypofibrinogenemia did not change during CBZ treatment, x indicates fibrinogen levels below the limit of detection (<0.5 g/L). (E) Monitoring of serum CBZ levels showed levels below the therapeutic window recommended for anticonvulsant therapy. (F) Decline of M65 and (G) M30 during CBZ treatment. d0 = start of CBZ; light blue box, duration of CBZ treatment.

autophagocytic vacuoles. Material of F2 III-1 showed late stage autophagocytic vacuoles with aggregates as cargo and electron dense multilamellar structures indicative of previous lysosomal fusion (degradative autolysosomes) (Fig. 1F and G). Quantitative morphometry showed a 5.15 increase in density of autophagocytic vacuoles in FSD (F2 III-4) and 2.81 increase in one ATD patient compared to normal/near normal pediatric liver biopsies (Supplementary data).

## Fibrinogen gamma mutations of probands and relatives

**Family 1:** Sequencing detected a heterozygous CGG → TGG transition at codon 375 in exon 9 of the *FGC* gene (fibrinogen Aguadilla) in the proband and in five additional family members in three generations (Fig. 2A, Supplementary data).

**Family 2:** Sequencing detected a heterozygous GGG → AGG transition in exon 8 in the proband (F2 III-1) and his father (F2 II-2). This transition causes a Gly → Arg substitution at codon 284

(fibrinogen Brescia). This mutation differs from the GGG → CGG transversion that was found in the only other fibrinogen Brescia family described so far [9]. Mutations were not detected in the grand-parents (F2 I-1 and F2 I-2) of the proband, indicative of a *de novo* mutation appearing in the father (Fig. 2B, Supplementary data).

## Clinical assessment of affected family members

Liver disease had not been suspected in any family member except the probands prior to this investigation. All affected family members showed hypofibrinogenemia (Supplementary data).

## Transient elastography

None of the patients exceeded normal range cut-off values, therefore providing no evidence of significant liver fibrosis (Supplementary data).

## CBZ treatment

Oral CBZ treatment led to rapid normalization of ALT levels (Fig. 2C) and aspartate aminotransferase (AST) levels (Supplementary data) in F1 III-4 and F2 III-1. Time to normalization was 35 and 26 days for F1 III-4 and F2 III-1, respectively. No significant change in fibrinogen levels and international normalized ratio (INR) levels were observed during CBZ treatment (Fig. 2D, Supplementary data). Levels of serum albumin, bilirubin,  $\gamma$ -glutamyltransferase, and alkaline phosphatase remained unchanged (not shown). The levels of surrogate markers for apoptosis and necrosis, M30 and M65 steadily decreased during treatment (Fig. 2G and F). M30 and M65 levels did not differ significantly from healthy controls (M30:  $141 \pm 33$ ; M65  $359 \pm 63$ , mean  $\pm$  standard error of the mean). After discontinuation of CBZ treatment, the effects on ALT, M30, and M65 reversed (Fig. 2C, G and F). Serum CBZ concentrations when receiving maintenance doses were  $1.5\text{--}4.5 \mu\text{g ml}^{-1}$  (median  $3.35$ ) for F1 III-4 (the level of  $1.5 \mu\text{g ml}^{-1}$  leading to dose adjustment from  $2 \times 140 \text{ mg d}^{-1}$  to  $2 \times 200 \text{ mg d}^{-1}$ ). CBZ levels for F2 III-1 were  $3.2\text{--}7.0 \mu\text{g ml}^{-1}$  (median  $4.45 \mu\text{g ml}^{-1}$ ) (Fig. 2E). Liver stiffness measurements performed on F1 III-4 and F2 III-1 during treatment (days 104 and 219 in F1 III-4 and days 111 and 208 in F2 III-1) showed unchanged non-pathological results (4.95 kPa and 4.7 kPa in F1 III-4; 4.5 kPa and 5.5 kPa in F2 III-1).

## Discussion

With only 26 affected individuals reported in the English literature, FSD qualifies as an orphan disease rendering controlled randomized trials unfeasible. The rationales justifying tentative uncontrolled and non-blinded initiation of CBZ treatment were: (i) the salient analogies with ATD, in particular increase in autophagy, (ii) evidence of persistent hepatocellular damage documented by liver biopsy and elevated serum amino-transferase in 2 out of 8 newly described patients, (iii) availability of a drug with a low-risk profile, whose effect on autophagy is well characterized in *in vitro* and *in vivo* studies, (iv) absence of causative treatment for liver disease in FSD. We considered the risk of CBZ-related toxicity to be low and outweighed by the advantages of a possible new treatment option for liver disease in FSD. In direct comparison with rapamycin, CBZ showed a better clearance of insoluble aggregates [4].

Both patients treated with CBZ showed rapid normalization of serum amino-transferase levels in combination with declining levels of M30 and M65 (exceeding M30 decline). This suggests reduction of apoptotic as well as necrotic hepatocellular death, both of which were observed in the diagnostic pretreatment liver biopsy of F1 III-4 (Fig. 1B and C) [8]. M30 and M65 levels before treatment were not significantly different from healthy individuals, which is in keeping with the subtle histological changes and absence of significant fibrosis in the biopsies.

Normalization of liver function has been reported in FSD and ATD following treatment with ursodeoxycholic acid (FSD) or  $\alpha$ -tocopherol and ursodeoxycholic acid (ATD) [10,11]. In these studies, ALT and other parameters returned to normal levels over a period of two years. In contrast, in our study ALT normalization occurred within 4 and 5 weeks of treatment and *vice versa*, suggesting a specific and direct mode of action. These may be mediated by selective autophagocytic clearance of ER aggregates and subsequent reduction of aggregate mediated toxicity analo-

gous to *in vitro* and *in vivo* models [4,5,12]. In addition, upregulation of non-selective autophagy has been demonstrated to exalt resistance against pro-apoptotic insults via enhanced clearance of mitochondria and subsequent reduction of cytosolic cytochrome c release [13]. This leads to reduction of downstream caspase activity, which could explain reduced M30 levels during treatment.

The stimulatory effect of CBZ on autophagy is dose dependent, does not affect synthesis or secretion of mutant protein, and is capable of further enhancing already upregulated autophagy as seen in our patients [4]. CBZ was also shown to enhance the proteasomal degradation of soluble mutant protein in addition to autophagy mediated clearance [4]. Interestingly, CBZ doses below the therapeutic window recommended for anticonvulsant therapy ( $6\text{--}12 \mu\text{g ml}^{-1}$ ) were sufficient to diminish liver damage in our patients.

The mechanisms by which accumulated mutant proteins mediate cellular toxicity in FSD are likely to be similar to ATD [6]. In mouse models, accumulation of polymeric mutant alpha1-antitrypsin Z causes ER stress, mitochondrial damage, activation of both pro-apoptotic, as well as regenerative pathways and activation of the autophagocytic response [6,14]. Hepatocellular injury promotes fibrosis via stellate cell activation [15]. The *in vivo* study in transgenic PiZ mice showed reduced levels of stellate cell activation markers and markedly decreased fibrosis in CBZ treated animals [4]. Therefore, decline of hepatocellular injury illustrated by ALT, M30, and M65 levels in our FSD patients indicates attenuated fibrogenic stimuli during CBZ therapy. Most FSD patients with significant fibrosis present in later life (Supplementary data). This suggests that progression of fibrosis in FSD is slow, hampering assessment of treatment effects on progression of fibrosis.

In conclusion, we provide evidence for feasibility of therapeutic exploitation of pharmacological enhancement of autophagy in FSD, which may also apply to other diseases characterized by aggregated proteins.

## Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jhep.2013.05.018>.



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